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Research report

Immunocytochemical demonstration of developmental distribution of muscarinic acetylcholine receptors in rat parietal cortex

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Abstract

The present investigation reveals many cortical neurons immunopositive for M35, the monoclonal antibody raised against purified muscarinic acetylcholine receptor (mAChR) proteins, in the early postnatal rat brain. The ontogeny of mAChR expression, exemplified on the parietal neocortex, was studied in a series of rat pups from postnatal days (PD) 1, 3, 7, 14 and 21. Immunoprecipitation in the parietal somatosensory cortex was manifest in the population of pyramidal neurons during postnatal development. In particular during the early postnatal ages, until 2 weeks after birth, M35 immunoreactivity (M35-ir) was present in all neuronal compartments, indicating transportation of mAChR protein in axonal and dendritic processes as observed in light and electron microscopic analysis. The immunoprecipitation in the apical dendrites yielded dense labeling in layer 1 where the distal processes of the pyramidal dendrites branched extensively forming a plexus that intermingled with horizontal fibers in this superficial layer. At PD21, immunolabeling in layer 1 and in axons of pyramidal cells was reduced compared to earlier ages suggesting a transient expression of mAChRs in these neuronal structures. The development of M35-ir in the cortex appeared to antedate that of its cholinergic afferentation as indicated by AChE histochemical study.

Keywords: Central nervous system; Cortical development; Muscarinic acetylcholine receptor; Acetylcholinesterase

1. Introduction

Neurotransmitters not only encode information, they also play an important role in the establishment of neuronal cytoarchitecture [18]. One of the transmitter systems that have a more general impact on the functioning and development of the mammalian telencephalon is the cholinergic innervation from the basal forebrain, which for this reason has received considerable attention. The anatomical and functional development of pre-and postsynaptic cholinergic elements has been studied with various biochemical and histological methods [2,6,8,9,12,13,14,15,17,20,21,28]. The ontogeny of cholinergic innervation of the cerebral cortex was mainly investigated histochemically, visualizing the degradative enzyme acetylcholinesterase (AChE) [5,8, 12], while the postsynaptic cholinergic development was predominantly directed towards the muscarinic receptor. For the mapping of muscarinic acetylcholine receptors (mAChR) in the brain radiolabeled agonists and antagonists have been used in biochemical binding studies [9,14,15,19,21,24]. More recently, the distribution of muscarinic receptor genes in the developing rat brain was visualized with in situ hybridization histochemistry [20]. The results of both techniques, however, are limited by the relatively low resolution level, decreasing the ability to identify morphological detail. This restriction currently can be avoided in immunohistochemical demonstration of receptor proteins. Antibodies have been raised against purified cholinergic receptors [1] and were used recently to demonstrate nicotinic [23] as well as muscarinic receptors [25,26] in the brain. The use of antibodies not only provides insight on the cellular and subcellular distribution of receptor proteins by increasing the detection level. A

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major additional advantage of immunocytochemical demonstration of receptor molecules, and not only the neurotransmitter or ligand binding site, is the information on intracellular transport mechanisms and distribution of receptor proteins in cell bodies as well as in axonal and dendritic extensions. In the present study the postnatal development of distribution of mAChR-proteins in rat parietal somatosensory cortex is visualized by light- and electron microscopic techniques, employing a monoclonal antibody, M35, raised against purified bovine forebrain mAChR-proteins [1]. The distribution of muscarinic receptors is compared with the cholinergic innervation of the cortex as shown by AChE histochemistry.

2. Materials and methods

2.1. Animals

Wistar dams were housed in a temperature- and light-controlled room (21 ± 1°C; 12/12 h light/dark cycle, light on at 7.00 a.m.). The day of birth of the offspring was taken as postnatal day (PD) 1. Within the first 2 days after delivery the litters were reduced to 8 per nest. Rats of either sex were sacrificed at PD 1, 3, 7, 14 and 21. For each age at least 4 brains were used. To an age group maximally two pups were used per nest to reduce the effect of litter.

2.2. Tissue preparation

Animals were deeply anesthetized with sodium pentobarbital. Fixation of the brain was carried out by transcardial perfusion with

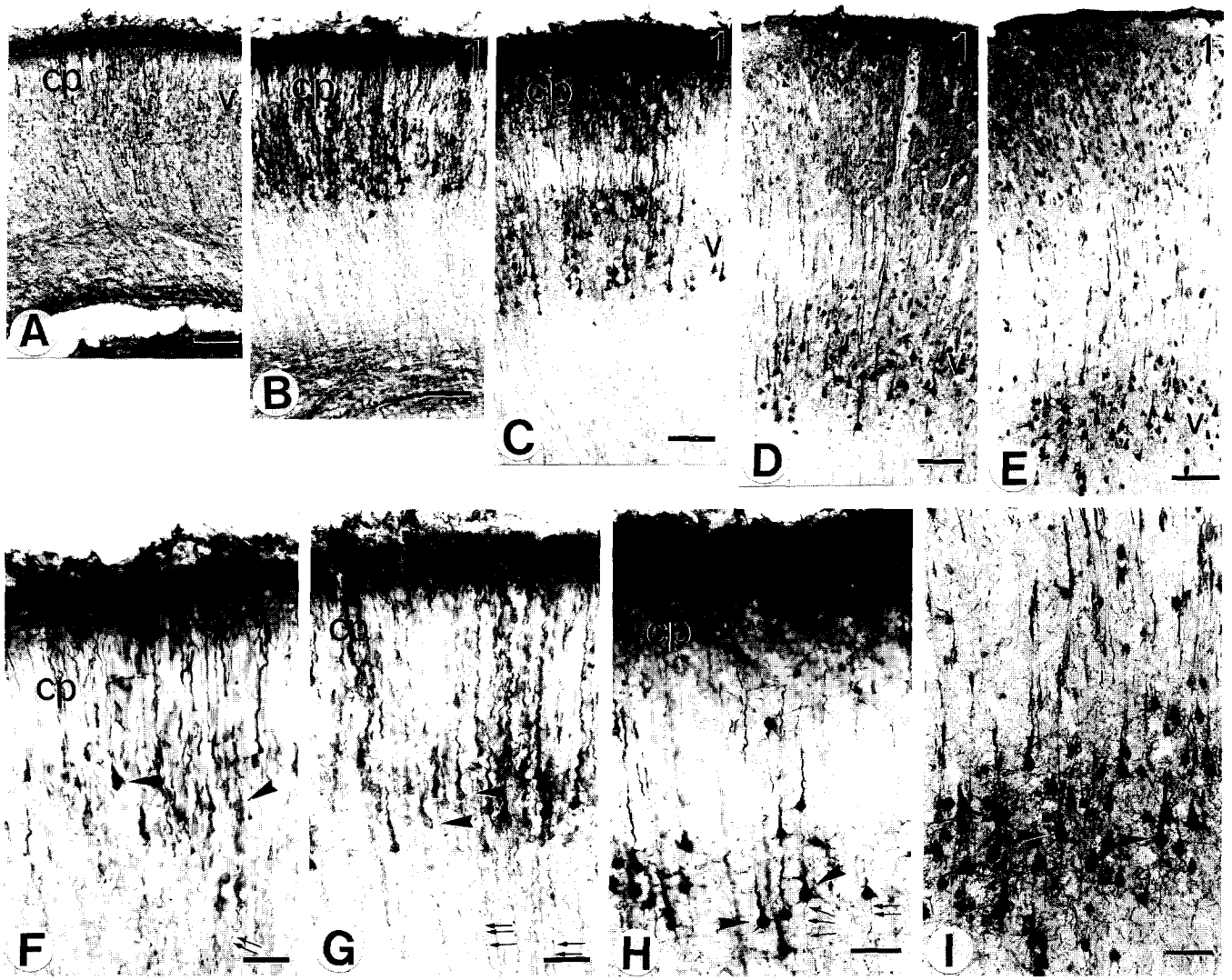

Fig. 1. Photomicrographs showing the development of M35-ir in rat parietal somatosensory cortex at postnatal days (PD) 1, 3, 7, 14 and 21 (A to E, respectively). Note the dense labeling in layer 1 at PD1 to 14. Pyramidal cells in the cortical plate (CP) are immunopositive already early postnatally, and will probably migrate to layer 5 in adulthood. F–I reveal detailed distribution of M35-ir in cell bodies and neurites at PD1 (F), 3 (G), 7 (H) and 21 (I). Arrowheads point to neuronal cellbodies. Axonal labeling is prominent in early postnatal ages (arrows). Scale bar in A–E = 100 μm, in F–I = 50 μm.

150 ml fixative composed of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4) at various perfusion speeds (7–16 ml/min), depending on the age and size of the animals. Fixation was preceded by a short rinse of saline. Immediately after the fixation the brain was removed, cryoprotected by overnight storage at 4°C in 30% sucrose in 0.1 M PB and subsequently coronally sectioned on a vibratome at a thickness of 20–40 μm.

Muscarinic receptors were visualized with the monoclonal antibody M35 against purified muscarinic receptor proteins [1]. For mAChR immunocytochemistry sections were pretreated with 0.1% H_2O_2 and 5% normal rabbit serum, immediately followed by incubation with M35 (mouse IgM) overnight at 4°C. After rinsing in phosphate buffered saline (PBS) (0.01 M; pH 7.4) and incubation in 5% normal rabbit serum, the sections were exposed to biotinylated rabbit anti-mouse IgM for 4 h at room temperature. Following overnight rinsing, sections were incubated with streptavidin conjugated HRP for 2 h at room temperature. All incubations took place in PBS without addition of detergents. HRP labels were routinely stained with DAB and H_2O_2 in Tris-HCl buffer (0.05 M; pH 7.6).

For AChE histochemistry, free-floating sections were postfixed for 12–24 h in 2.5% glutaraldehyde in 0.05 M PB and stained according to the procedure as described by Hedreen et al. [7].

Subcellular electron-microscopic investigation of M35 distribution was carried out by postfixation of immunostained sections with 1% osmium tetroxide in 0.1 M PB for 1 h, dehydration in an ascending series of ethanol, and subsequent staining en bloc with 1% uranyl acetate in 70% ethanol. Thereafter, the sections were flat embedded in Epon. Ultrathin sections were prepared on an LKB ultramicrotome and collected on 200-mesh grids. Grids were contrasted with Reynolds lead solution. Sections were examined in a Philips 201 electron microscope.

3. Results

3.1. Light microscopic observation of M35 immunostaining and AChE histochemistry

Most noticeable was the prominent immunoprecipitation in pyramidal cell bodies and apical dendrites

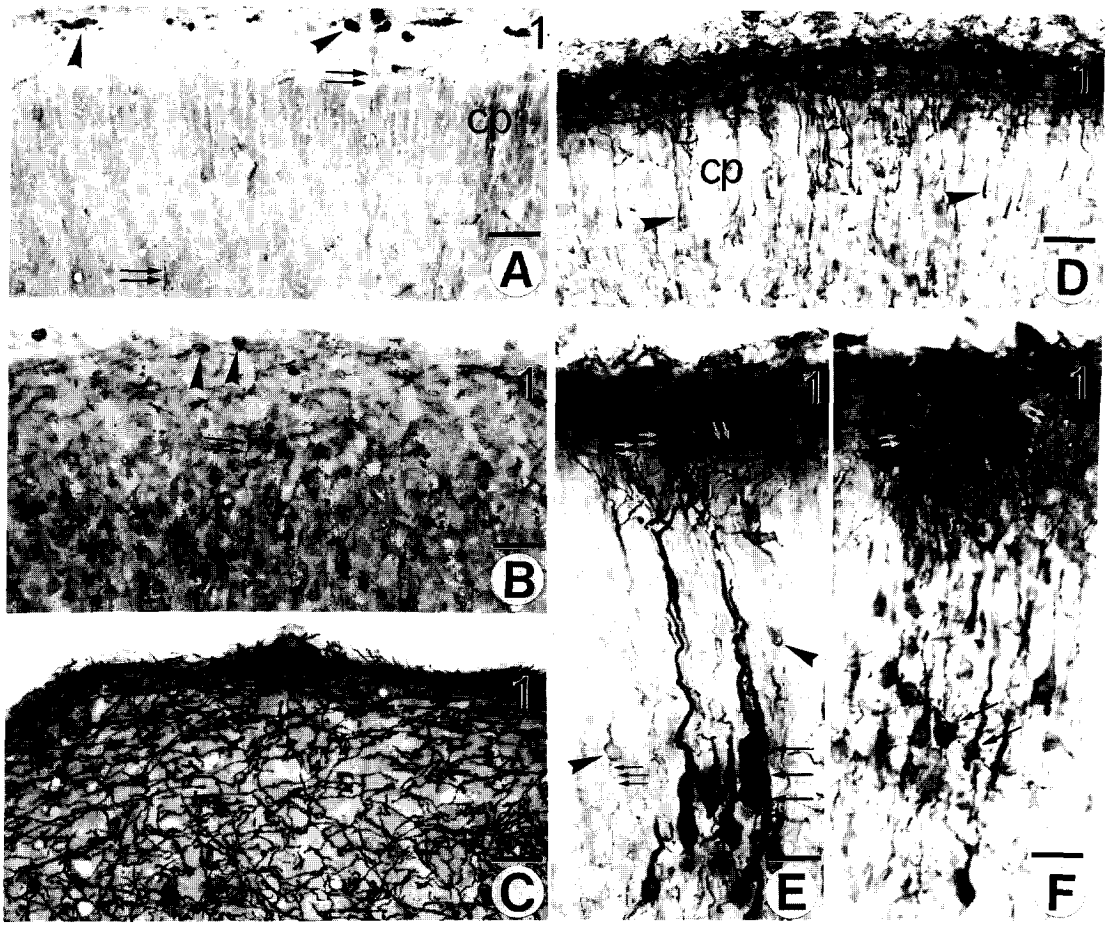

Fig. 2. (A–C) Upper cortical layers at PD1 (A), PD7 (B) and PD21 (C) showing AChE-positive fibers. In superficial layer 1 AChE positive Retzius-Cajal cells can be observed at PD1 and 7 (arrowheads). In the early postnatal period very few fibers (arrows) reach the cortical plate (CP) and the upper layer of the cortex. This number is rapidly increasing at PD7 (B) and mature at PD21 (C). D reveals detailed picture of M35-ir in the upper cortical layer 1 and CP at PD1. Fibers can be observed crossing horizontally through layer 1 together with dendritic branches. Weakly differentiated immunopositive neuronal cellbodies are located in the CP (arrowheads). E and F compare the cytological differentiation at PD1 (E) and PD7 (F) of M35-ir pyramidal cells in the CP. Large arrows indicate neuronal somata neighboring in radial rows at PD1 showing little cellular differentiation. Some weaker stained cell bodies can be observed (arrowheads) with descending immunopositive axons (small arrows). Apical dendrites branch in layer 1 building dendritic trees (white arrows). Notice the advanced cellular differentiation at PD7 when basal dendrites start to develop (arrows). Scale bars in A–D = 50 μm; E, F = 25 μm.

throughout postnatal development. With increasing age a clear laminar distribution of M35 immunostaining in the cortex started to appear (Fig. 1A–E). In the cortical plate (CP) of early postnatal ages PD1, 3 and 7, pyramidal neurons were immunopositive. The process of migration of these pyramidal neurons from the CP to their final position in layer 5 of the cortex at PD21 is demonstrated in Fig. 1A–E. At PD1, M35-immunoreactive (M35-ir) pyramidal neurons at various levels of the CP were in close apposition, sometimes neighboring in couples or lined up in radial rows (Fig. 1F and 2E). Neuronal somata and short erect apical dendrites showed little cytological differentiation the day after birth. Only deeper pyramidal cells in the CP were beginning to grow faint immunostaining in basal dendrites (Fig. 2E). A further striking observation was the dense precipitation early postnatally in the superficial marginal zone or layer 1. The intensity of staining in this layer decreased 2 weeks after birth and was relatively weak at PD21 (Fig. 1A–H). The labeling in layer 1 was mainly caused by the apical tufts of pyramidal neurons in the CP forming a dense plexus (Fig. 2D–F). These M35-ir dendritic branches intermingled with horizontally crossing fibers, possibly axonal processes coming from immunopositive interneurons in the subplate, yielding the dense M35 immunoprecipitate in layer 1 during the first week of postnatal development. At PD3, pyramidal neurons were not anymore in apposition to each other indicating the process of ongoing migration within the CP. Fig. 1B also shows a clear division at PD3 between CP and subplate which is less visible at PD1 (Fig. 1A). Descending immunoreactive axons originating from pyramidal neurons positioned in the CP were traversing radially through the subplate (Fig. 1F,G). Although not recognizable in the photomicrographs, many M35 positive multipolar neurons were observed in the subplate at early postnatal ages. Two and three weeks after birth these multipolar neurons were observed throughout layers 2 to 6. At PD7 the laminar cortical distribution of M35-ir became manifest due to the migration of neurons towards their final positions in cortical layers (Fig. 1C). The pyramidal cells showed somatic differentiation in the outgrowth of basal dendrites and many labeled processes between somata of future layer 5 (Figs. 1H and 2F). Fig. 2H also demonstrates the sharp staining of descending axons at PD7 indicating intra-axonal transport of mAChRs at this age. Two weeks after birth, the development of M35-ir approached the mature distribution pattern with immunoreactivity in layer 1 weakening compared to earlier ages. Pyramidal neurons in layer 2/3 revealed dense immunoprecipitation, while their descending axons were still immunopositive at PD14 (Fig. 1D). Fig. 1E and I show the distribution of M35 at PD21. At this age axonal staining was sparse and cytological differentiation of pyramidal somata apparently complete. Im-

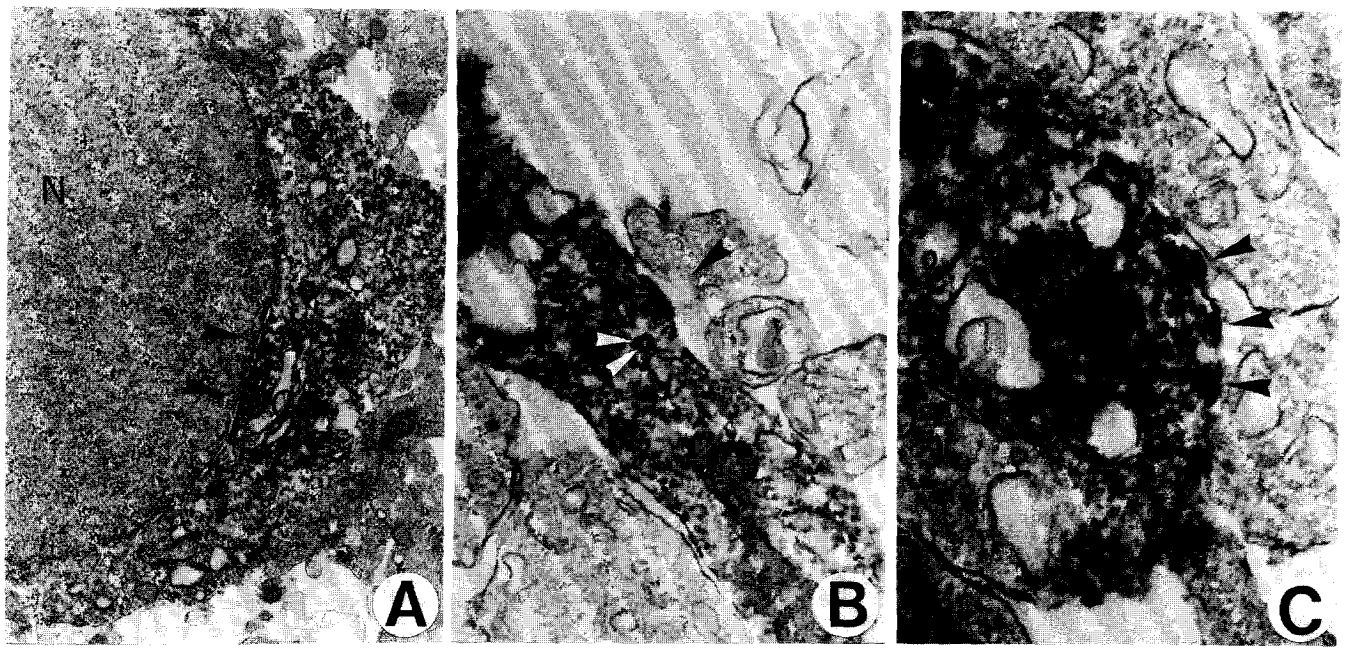

Fig. 3. Electron microscopic photomicrographs showing M35-ir neuronal somata and dendrites at PD3 in the cortical plate and layer 1. A: neuronal cell body with M35 immunoprecipitation around the unlabeled nucleus (N). Dense labeling can be observed adjacent to the cell's Golgi apparatus (arrowheads). B: M35-ir can be seen in a dendritic process running in upper layer 1. Note the immunoprecipitation associated with the exterior of vesicles (white arrowheads). Clusters of immunoreactivity are present in the plasma membrane (black arrowheads). C: dense dendritic labeling in the superficial layer 1. Clusters of M35-ir can be observed in the plasma membrane in the absence of presynaptic specializations.

munoreactivity in layer 1 was weak as in layer 4 which was almost devoid of precipitate except for traversing apical dendrites of layer 5 pyramidal cells.

Fig. 2A–C shows the development of cholinergic cortical innervation as demonstrated by the histochemical AChE staining procedure. At PD1 (Fig. 2A) very few cholinergic fibers reached the upper cortical layer and CP. Horizontally oriented AChE-positive cells, presumably Cajal-Retzius cells, were densely stained in layer 1. At PD7 (Fig. 2B) cholinergic fibers of the CP and layer 1 were present in increasing amounts. The Cajal-Retzius cells producing AChE were also observed. Three weeks after birth the AChE fiber distribution showed the adult innervation pattern (Fig. 2C).

3.2. Electron microscopic (EM) observations of M35-immunopositive staining

To study the early postnatal intracellular distribution of mAChRs, we focussed on immuno-EM precipitates in the cortical plate and layer I at PD3. M35 immunostaining was absent in the cellular nucleus but could be seen throughout the neuronal cytoplasm with dense patches of immunoreactivity around the Golgi-apparatus (Fig. 3A). EM observations further revealed precipitation present on the exterior side of cytoplasmic vesicles in dendritic processes (Fig. 3B,C). In the cellular membrane of dendrites immunoprecipitate could be seen in dense clusters, however, without the presence of apposing presynaptic nerve endings.

4. Discussion

The present investigation showed that in the early postnatal brain many cortical neurons were immunoreactive for the M35 monoclonal antibody raised against mAChR proteins. Dense immunoprecipitation in the parietal cortex was most strikingly manifest in the pyramidal neurons throughout postnatal development. In particular during the early postnatal ages until 2 weeks after birth, M35-ir throughout the cytoplasm of neuronal cell bodies indicated transportation of mAChRs in axonal and dendritic extensions. This transport of receptor proteins to axonal and dendritic endings caused dense transient labeling in layer 1 where pyramidal apical dendrites branched forming an immunopositive plexus. Horizontally running fibers, presumably axons originating from M35-ir multipolar interneurons located in the subplate traversed through this superficial layer. It is also possible that these fibers are processes of the horizontally oriented Cajal-Retzius cells, which revealed dense AChE staining during early postnatal development.

The development of M35-ir in the cortex appeared to precede that of its cholinergic afferentation as indicated by the AChE histochemical study. This confirms the results and conclusions in other studies [8,12]. It has been suggested that cholinergic afferents are involved in synaptic plasticity and cortical differentiation [3,10]. In neuromuscular junctions nicotinic acetylcholine receptors were shown to be present on muscle cells prior to the formation of synaptic contacts [11]. It was sugggested in that study that AChR accumulations in muscle membrane might be involved in the initiation and guidance of synapse formation by the growing motor axon. The receptor positive surface patches on myotubes resemble the M35-ir patches on the dendrites in the cortex as visualized in our EM observations. Particularly during early postnatal development, but until PD14, transportation of mAChRs was suggested by M35-ir in somata and in both dendritic and axonal extensions. The neuronal growth cone, the motile tip of growing neurites, is considered to be responsible for guidance of axons and dendrites and for future synaptic transmission [4,27]. During the early postnatal development dendrites are branching massively in superficial layer 1 building complex dendritic trees while axons are running horizontally in this layer possibly making synaptic contacts. Growth cones are intimately involved in the outgrowth of dendritic and axonal neurites. Until 2 weeks postnatally, presynaptic immunoprecipitation was also observed in many descending pyramidal axons. Although in adult animals little presynaptic M35-ir is observed, behaviorally activated pyramidal neurons in cortex were characterized by M35 immunoprecipitation in axons [26], suggesting that presynaptic muscarinic receptors are involved in processes occurring during behaviorally induced synaptic plasticity. The dense M35-ir labeling in layer 1 and in descending pyramidal axons in early postnatal development might indicate a role for mAChRs in growth cone related pathfinding. It has been shown that neurotransmitters can act as morphogenic growth regulators in the developing central nervous system [16,18]. Furthermore, mAChRs are reported to be expressed and more concentrated in growth cone membranes than in any other membrane portion isolated from fetal and neonatal rat forebrain [22]. Zheng et al. [29] recently showed that AChRs are involved in guidance and extension rate of the growth cone. The phosphorylation of growth cone B50 proteins, involved in neuritic outgrowth, by muscarinic receptor activation possibly plays an important role in these processes [27]. At PD21, when developmental growth processes in the brain are nearing completion, and in adult animals (see 25), presynaptic labeling as well as immunoprecipitation in dendritic branches were sparse.

The ontogenic distribution of cortical mAChRs as described in this study is in line with some previous reports, but also partly differs from some biochemical ligand studies. There probably are a number of factors

contributing to different findings in ligand binding and immunolabeling studies. M35 apparently does not discriminate between different mAChR subtypes [25], recognizing an epitope on the muscarinic receptor protein which permits visualization of mAChR protein in the cytoplasm. This in contrast with ligand binding studies showing only the agonist or antagonist binding sites of the functional receptor in its synaptic intramembranous configuration. Furthermore, differences in findings appear to be dependent on the nature of the ligand used in autoradiography. Muscarinic receptor binding studies with Scatchard analysis applying tritiated methylscopolamine and quinuclinidyl benzilate (^{3}[H]NMS and ^{3}[H]QNB) showed a rather linear increase in muscarinic B_{max} value during subsequent maturation of the brain [2,15,17,19]. Studies in which the irreversible muscarinic antagonist [^{3}H]propylbenzilylcholine mustard ([^{3}H]PrBCM) was used for autoradiographic localization of mAChRs showed a nonlinear cortical radioligand labeling during development with a peak density on PD10 [21]. This antagonist further showed, like the M35 antibody, the transient labeling in cortical layer 1 [9,21]. In situ hybridization histochemistry to reveal the expression of m1, m3 and m4 mAChRs in the developing rat visual cortex confirmed the present findings, indicated a relatively high gene expression in upper cortical layers during early postnatal development [20].

The present study shows that mAChRs are numerous in early postnatal cortical development. The transient appearance of these receptors in dendritic and axonal endings suggest a role in cytological differentiation and extension of processes.

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